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(21) International Application Number: PCT/DK95/00384 (22) International Filing Date: 27 September 1995 (27.09.95) (30) Priority Data: 1109/94 27 September 1994 (27.09.94) DK 0952/95 25 August 1995 (25.08.95) DK 1044/95 19 September 1995 (19.09.95) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): SCHNEIDER, Palle [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). DAMHUS, Ture [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ENHANCERS SUCH AS ACETOSYRINGONE (57) Abstract The present invention relates to a method of oxidizing a compound with a phenol oxidizing enzyme (e.g. a peroxidase or a laccase) and an enhancing agent (e.g. acetosyringone). The invention also relates to a detergent additive and to a detergent composition.		

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ENHANCERS SUCH AS ACETOSYRINGONE

FIELD OF INVENTION

The invention relates to a method of oxidizing a compound with a phenol oxidizing enzyme and an enhancing agent. The invention also relates to a detergent additive and to a detergent composition.

BACKGROUND ART

By a phenol oxidizing enzyme is meant an enzyme which by using hydrogen peroxide or molecular oxygen, is capable of oxidizing organic compounds containing phenolic groups. Examples of such enzymes are peroxidases and oxidases.

It has earlier been found that coloured substances leached from dyed fabrics could be bleached by means of a phenol oxidizing enzyme. The use of peroxidases or oxidases for inhibiting dye transfer in this way is described in WO 91/05839.

Certain oxidizable substances, e.g., metal ions and phenolic compounds such as 7-hydroxycoumarin, vanillin, and p-hydroxybenzenesulfonate, have been described as accelerators or enhancing agents able to enhance enzymatic bleaching reactions (cf. e.g. WO 92/18683, WO 92/18687, and Kato M and Shimizu S, Plant Cell Physiol. 1985 **26** (7), pp. 1291-1301 (cf. Table 1 in particular)). In WO 94/12621 other types of enhancing agents are disclosed, e.g., phenothiazines and phenoxazines.

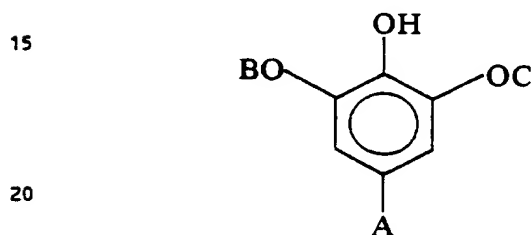
It is the object of this invention to provide a new group of enhancing agents which are effective for enhancing phenol oxidizing enzymes.

SUMMARY OF THE INVENTION

It has now surprisingly been found that a new group of organic chemical substances performs excellently as enhancers of phenol oxidizing enzymes.

5 This new group of organic chemical substances not only make the bleaching reactions faster compared with using the phenol oxidizing enzyme alone, but many compounds which could not be bleached at all, may now be bleached by using the method of the invention.

10 Accordingly, the invention provides a method of oxidizing a compound with a phenol oxidizing enzyme, characterized by the presence of an enhancing agent of the following formula:



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, -SO₂-E, -N-XY, and -N⁺-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y
 25 and Z may be identical or different and selected from -H and -R; R being a C₁-C₁₆ alkyl, preferably a C₁-C₈ alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected
 30 from C_mH_{2m+1}; 1 ≤ m ≤ 5.

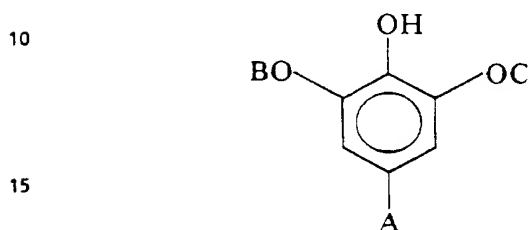
BRIEF DESCRIPTION OF THE DRAWING

The present invention is further illustrated by reference to Fig. 1 which shows the bleaching of gradually added Acid Blue 45 in phosphate/borate buffer pH 10 at 35°C;

(I): Only dye addition; (II): Dye addition in the presence of Laccase; (III): Dye addition in the presence of Laccase + Acetosyringone; the experiment conducted as described in Example 8.

5 DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of oxidizing a compound with a phenol oxidizing enzyme, characterized by the presence of an enhancing agent of the following formula:



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, -SO₂-E, -N-XY, and
 20 -N⁺-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C₁-C₁₆ alkyl, preferably a C₁-C₈ alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino
 25 group; and B and C may be the same or different and selected from C_mH_{2m+1}; 1 ≤ m ≤ 5.

In a preferred embodiment A in the above mentioned formula is -CO-E, in which E may be -H, -OH, -R, or -OR; R being a C₁-C₁₆ alkyl, preferably a C₁-C₈ alkyl, which alkyl may
 30 be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m+1}; 1 ≤ m ≤ 5.

In the above mentioned formula A may be placed meta to the hydroxy group instead of being placed in the paraposition as shown.

In particular embodiments, the enhancing agent is acetosyringone, syringaldehyde, methylsyringate, syringic acid, ethylsyringate, propylsyringate, butylsyringate, hexylsyringate, octylsyringate or ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate.

The enhancing agent of the invention may be present in concentrations of from 0.01 to 1000 μM , more preferred 0.1 to 250 μM , most preferred 1 to 100 μM .

Preparation of Enhancing Agents

The enhancing agents described in the present application may be prepared using methods well known to those skilled in the art; some of the enhancing agents are also commercially available.

We produced methylsyringate, ethylsyringate, propylsyringate, butylsyringate, hexylsyringate and octylsyringate by using the method disclosed in Chem. Ber. 67, 1934, p. 67.

Ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate was synthesised from syringaldehyde and triethyl phosphonoacetate in ethanol/sodium ethanolate. The product was after purification characterised by ^1H -NMR and ^{13}C -NMR (showing spectra as expected) and the melting point was 68-70°C.

Hydrogen peroxide/Oxygen

If the phenol oxidizing enzyme requires a source of hydrogen peroxide, the source may be hydrogen peroxide or a hydrogen peroxide precursor for in situ production of hydrogen peroxide, e.g., percarbonate or perborate, or a hydrogen peroxide generating enzyme system, e.g., an oxidase and a substrate for the oxidase, e.g., an amino acid oxidase and a suitable amino acid, or a peroxy-carboxylic acid or a salt thereof. Hydrogen peroxide may be added at the beginning

or during the process, e.g., in an amount corresponding to levels of from 0.001-25 mM, particularly to levels of from 0.01-1 mM.

If the phenol oxidizing enzyme requires molecular oxygen, molecular oxygen from the atmosphere will usually be present in sufficient quantity. If more O₂ is needed, additional oxygen may be added.

Phenol Oxidizing Enzyme

In the context of the present invention the enzyme of the phenol oxidizing enzyme may be an enzyme possessing peroxidase activity or a laccase or a laccase related enzyme as described below.

Peroxidases and Compounds possessing Peroxidase Activity

Compounds possessing peroxidase activity may be any peroxidase enzyme comprised by the enzyme classification (EC 1.11.1.7), or any fragment derived therefrom, exhibiting peroxidase activity, or synthetic or semisynthetic derivatives thereof (e.g. porphyrin ring systems or microperoxidases, cf. e.g. US Patent 4,077,768, EP Patent Application 537,381, International Patent Applications WO 91/05858 and WO 92/16634).

Preferably, the peroxidase employed in the method of the invention is producible by plants (e.g. horseradish or soybean peroxidase) or microorganisms such as fungi or bacteria. Some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g. Fusarium, Humicola, Trichoderma, Myrothecium, Verticillium, Arthromyces, Caldariomyces, Ulocladium, Embellisia, Cladosporium or Dreschlera, in particular Fusarium oxysporum (DSM 2672), Humicola insolens, Trichoderma resii, Myrothecium verrucaria (IFO 6113), Verticillium alboatrum, Verticillium dahliae, Arthromyces ramosus (FERM P-7754), Caldariomyces fumago, Ulocladium chartarum, Embellisia allior Dreschlera halodes.

Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g. Coprinus, Phanerochaete, Coriolus or Trametes, in particular Coprinus cinereus f. microsporus (IFO 5 8371), Coprinus macrorhizus, Phanerochaete chrysosporium (e.g. NA-12) or Trametes (previously called Polyporus), e.g. T. versicolor (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, 10 e.g. Rhizopus or Mucor, in particular Mucor hiemalis.

Some preferred bacteria include strains of the order Actinomycetales, e.g. Streptomyces spheroides (ATCC 23965), Streptomyces thermoviolaceus (IFO 12382) or Streptoverticillum verticillium ssp. verticillium.

15 Other preferred bacteria include Bacillus pumilus (ATCC 12905), Bacillus stearothermophilus, Rhodobacter sphaeroides, Rhodomonas palustri, Streptococcus lactis, Pseudomonas purrocinia (ATCC 15958) or Pseudomonas fluorescens (NRRL B-11).

20 Further preferred bacteria include strains belonging to Myxococcus, e.g. M. virescens.

The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA 25 sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase, in a culture medium under conditions permitting the expression of the peroxidase and recovering the peroxidase from the culture.

30 Particularly, a recombinantly produced peroxidase is a peroxidase derived from a Coprinus sp., in particular C. macrorhizus or C. cinereus according to WO 92/16634.

In the context of this invention, compounds 35 possessing peroxidase activity comprise peroxidase enzymes and peroxidase active fragments derived from cytochromes, haemoglobin or peroxidase enzymes, and synthetic or

semisynthetic derivatives thereof, e.g., iron porphyrins, and iron phthalocyanines and derivatives thereof.

Determination of Peroxidase Activity (PODU)

1 peroxidase unit (PODU) is the amount of enzyme that catalyzes the conversion of 1 μ mole hydrogen peroxide per minute at the following analytical conditions: 0.88 mM hydrogen peroxide, 1.67 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), 0.1 M phosphate buffer, pH 7.0, incubated at 30°C, photometrically followed at 418 nm.

10 Laccase and Laccase Related Enzymes

In the context of this invention, laccases and laccase related enzymes comprise any laccase enzyme comprised by the enzyme classification (EC 1.10.3.2), any catechol oxidase enzyme comprised by the enzyme classification (EC 1.10.3.1), any bilirubin oxidase enzyme comprised by the enzyme classification (EC 1.3.3.5) or any monophenol monooxygenase enzyme comprised by the enzyme classification (EC 1.14.99.1).

The above mentioned enzymes may be derived from plants, bacteria or fungi (including filamentous fungi and yeasts) and suitable examples include a laccase derivable from a strain of Aspergillus, Neurospora, e.g., N. crassa, Podospira, Botrytis, Collybia, Fomes, Lentinus, Pleurotus, Trametes, e.g., T. villosa and T. versicolor, Rhizoctonia, e.g., R. solani, Coprinus, e.g., C. cinereus, C. comatus, C. friesii, and C. plicatilis, Psathyrella, e.g., P. condelleana, Panaeolus, e.g., P. papilionaceus, Myceliophthora, e.g., M. thermophila, Schytalidium, Polyporus, e.g., P. pinsitus, Phlebia, e.g., P. radita (WO 92/01046), or Coriolus, e.g., C. hirsutus (JP 2-238885).

The laccase or the laccase related enzyme may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the laccase, in a

culture medium under conditions permitting the expression of the laccase enzyme, and recovering the laccase from the culture.

Determination of Laccase Activity (LACU)

5 Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19 μ M syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min. reaction time.

10 1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0 μ mole syringaldazin per minute at these conditions.

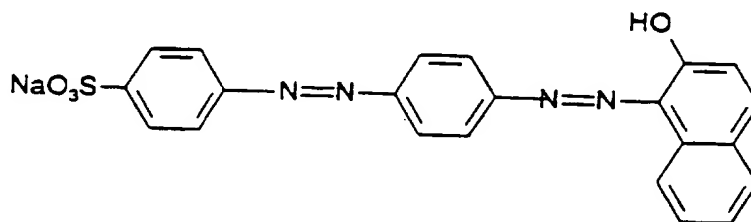
Industrial Applications

In a preferred embodiment, the method of the
15 invention finds application for bleaching of a textile dye or colorant or textile dyes or colorants in solution.

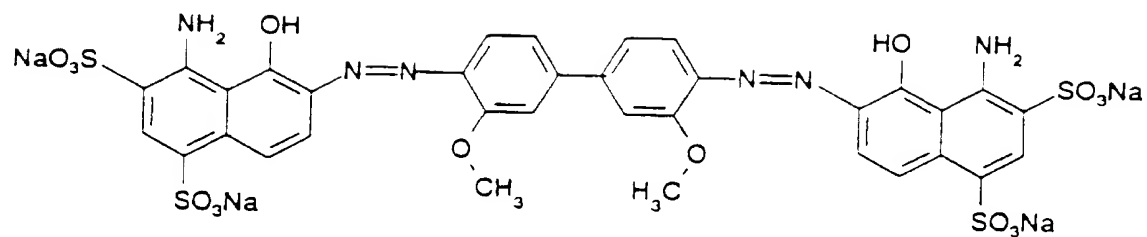
Colorants and dyes are broad classes of natural and synthetic compounds. The following description and examples of dyes/colorants are not intended to be in any
20 way limiting to the scope of the invention as claimed:

Synthetic textile dyes bleachable by the method of the invention are typically azo compounds (with one or several azo, or diazenediyl, groups), as exemplified by Acid Red 151, Direct Blue 1, Direct Brown 44, and Orange II,
25 or anthraquinone compounds, as exemplified by Acid Blue 45:

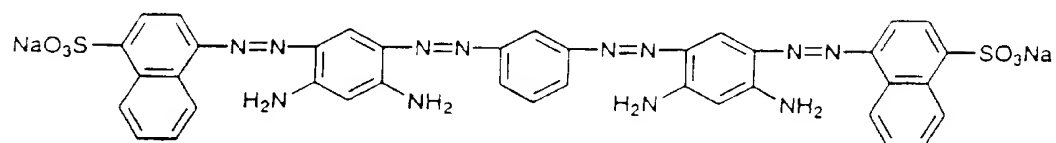
Acid Red 151



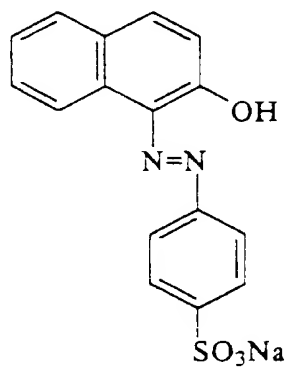
Direct Blue 1



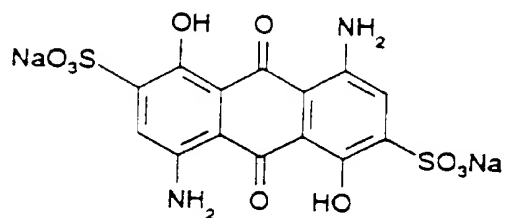
Direct brown 44



Orange II

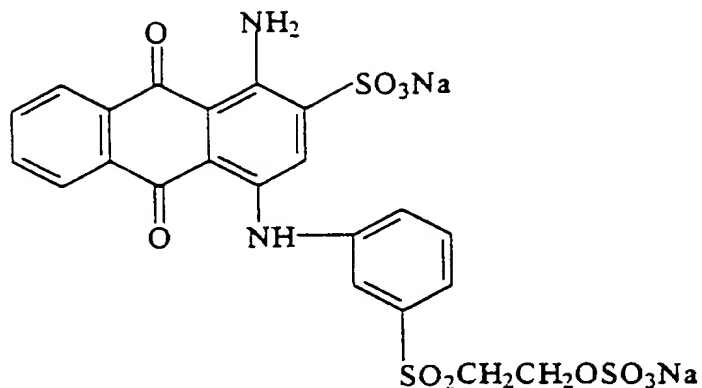


Acid Blue 45



Other structural motifs may occur together with these, as exemplified in the formula of Reactive Blue 19:

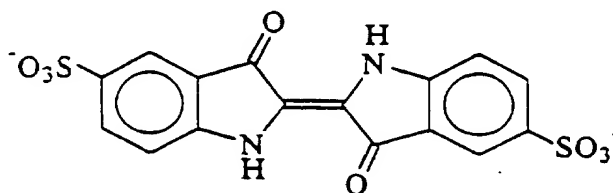
Reactive Blue 19



Some dyes furthermore carry groups capable of coupling to fabric surfaces (reactive dyes), and some dyes are complexed to metal ions. These modifications will often not influence the applicability of the present invention.

A different structure bleachable by the method of the invention is the indigo moiety, here exemplified by the soluble dye indigo carmine:

Indigo Carmine



Other dyes and colorants may be of natural origin or may be synthesized as identical to or resembling natural structures. Examples of categories of coloured substances extractable from vegetable sources are polyphenolic, anthocyanine and carotenoid compounds.

A specific embodiment of the present invention is provided by household and institutional laundering processes. In such washing and rinsing processes, dyes and colorants present on fabrics may leach into the washing or rinsing liquor and discoloration of the laundry may result. Bleaching

of the coloured compounds in solution by the method of the invention may counteract this undesirable effect. Other systems for dye transfer inhibition are known in the art (e.g. WO 91/05839).

5 In another specific embodiment, dyes leached into process water during textile processing may be bleached by the method of the invention to prevent undesirable deposition. Other systems are known in the art (e.g. WO 92/18697).

10 In a third embodiment, the method of the invention finds application in bleaching of pulp for paper production.

Accordingly, the invention provides a method for bleaching of lignin-containing material, in particular
15 bleaching of pulp for paper production, which method comprises treatment of the lignin or lignin containing material with a phenol oxidizing enzyme and an enhancing agent as described in the present invention.

In a fourth embodiment, the method of the
20 invention finds application for lignin modification, e.g., in the manufacture of wood composites, e.g., wood fibre materials such as chipboards, fibre boards, or particle boards, or in the manufacture of laminated wood products, such as laminated beams and plywood.

25 In a fifth embodiment, the method of the invention finds application in treatment of waste water, e.g., waste water from the chemical or pharmaceutical industry, from dye manufacturing, from dye-works, from the textile industry, or from pulp production (cf. e.g. US
30 4,623,465, or JP-A-2-31887).

In a more specific aspect, the invention provides a method for treatment of waste water from dye manufacturing, from dye-works, from textile industry, or from pulp manufacturing, the method comprising treatment of the
35 waste water with a phenol oxidizing enzyme in the presence of an enhancing agent of the invention.

In the above mentioned processes and in other applications of the invention, the enhancing agent may be

added at the beginning of the process or later, in one or several additions.

According to the invention the phenol oxidizing enzyme may be present in concentrations of from 0.001-100 mg enzyme protein per liter.

Detergent Compositions

According to the invention, the enhancing agent and the phenol oxidizing enzyme may typically be a component of a detergent composition. As such, it may be included in the
10 detergent composition in the form of a detergent additive. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as
15 disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonyl-
20 phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-
25 forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods.
30 Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, .g. as powder, granules, paste or
35 liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene-
5 sulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as
10 alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

15 The detergent composition may additionally comprise one or more other enzymes, such as amylases, lipases, cutinases, proteases, and cellulases.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate,
20 triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially
25 free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates,
30 maleic/acrylic acid copolymers and lauryl methacrylate/-acrylic acid copolymers.

The detergent may additionally contain other bleaching systems which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming
35 bleach activator such as tetraacetythylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g., in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

20	Linear alkylbenzenesulfonate (calculated as acid)	7	-	12%
	Alcohol ethoxysulfate (e.g. C ₁₂₋₁₈ alcohol, 1-2 EO) or alkyl sulfate (e.g. C ₁₆₋₁₈)	1	-	4%
25	Alcohol ethoxylate (e.g. C ₁₄₋₁₅ alcohol, 7 EO)	5	-	9%
	Sodium carbonate (as Na ₂ CO ₃)	14	-	20%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	2	-	6%
30	Zeolite (as NaAlSiO ₄)	15	-	22%
	Sodium sulfate (as Na ₂ SO ₄)	0	-	6%
	Sodium citrate/citric acid (as C ₆ H ₅ Na ₃ O ₇ /C ₆ H ₈ O ₇)	0	-	15%
	Sodium perborate (as NaBO ₃ ·H ₂ O)	11	-	18%
35	TAED	2	-	6%
	Carboxymethylcellulose	0	-	2%

	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
5	Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

10	Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
	Alcohol ethoxysulfate (e.g. C ₁₂₋₁₈ alcohol, 1-2 EO or alkyl sulfate (e.g. C ₁₆₋₁₈))	1 - 3%
15	Alcohol ethoxylate (e.g. C ₁₄₋₁₅ alcohol, 7 EO)	5 - 9%
	Sodium carbonate (as Na ₂ CO ₃)	15 - 21%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	1 - 4%
	Zeolite (as NaAlSiO ₄)	24 - 34%
20	Sodium sulfate (as Na ₂ SO ₄)	4 - 10%
	Sodium citrate/citric acid (as C ₆ H ₅ Na ₃ O ₇ /C ₆ H ₈ O ₇)	0 - 15%
	Carboxymethylcellulose	0 - 2%
25	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
	Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

30 3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
35	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO)	7 - 14%
	Soap as fatty acid (e.g. C ₁₆₋₂₂ fatty acid)	1 - 3%

	Sodium carbonate (as Na_2CO_3)	10	- 17%
	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$)	3	- 9%
	Zeolite (as NaAlSiO_4)	23	- 33%
	Sodium sulfate (as Na_2SO_4)	0	- 4%
5	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$)	8	- 16%
	TAED	2	- 8%
	Phosphonate (e.g. EDTMPA)	0	- 1%
	Carboxymethylcellulose	0	- 2%
10	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0	- 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
20	Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10	- 25%
	Sodium carbonate (as Na_2CO_3)	14	- 22%
	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$)	1	- 5%
	Zeolite (as NaAlSiO_4)	25	- 35%
25	Sodium sulfate (as Na_2SO_4)	0	- 10%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
30	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

5) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO or C ₁₂₋₁₅ alcohol, 5 EO)	12	- 18%
	Soap as fatty acid (e.g. oleic acid)	3	- 13%
	Alkenylsuccinic acid (C ₁₂₋₁₄)	0	- 13%
	Aminoethanol	8	- 18%
10	Citric acid	2	- 8%
	Phosphonate	0	- 3%
	Polymers (e.g. PVP, PEG)	0	- 3%
	Borate (as B ₄ O ₇)	0	- 2%
	Ethanol	0	- 3%
15	Propylene glycol	8	- 14%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0	- 5%

6) An aqueous structured liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	3	- 9%
	Soap as fatty acid (e.g. oleic acid)	3	- 10%
10	Zeolite (as NaAlSiO ₄)	14	- 22%
	Potassium citrate	9	- 18%
	Borate (as B ₄ O ₇)	0	- 2%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. PEG, PVP)	0	- 3%
15	Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	- 3%
	Glycerol	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0	- 5%

25 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Fatty alcohol sulfate	5	- 10%
	Ethoxylated fatty acid monoethanol-amide	3	- 9%
30	Soap as fatty acid	0	- 3%
	Sodium carbonate (as Na ₂ CO ₃)	5	- 10%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	1	- 4%
	Zeolite (as NaAlSiO ₄)	20	- 40%
	Sodium sulfate (as Na ₂ SO ₄)	2	- 8%
35	Sodium perborate (as NaBO ₃ ·H ₂ O)	12	- 18%
	TAED	2	- 7%

	Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
5	Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0	-	5%

8) A detergent composition formulated as a granulate comprising

10	Linear alkylbenzenesulfonate (calculated as acid)	8	-	14%
	Ethoxylated fatty acid monoethanolamide	5	-	11%
	Soap as fatty acid	0	-	3%
15	Sodium carbonate (as Na_2CO_3)	4	-	10%
	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$)	1	-	4%
	Zeolite (as NaAlSiO_4)	30	-	50%
	Sodium sulfate (as Na_2SO_4)	3	-	11%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	5	-	12%
20	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
25	Minor ingredients (e.g. suds suppressors, perfume)	0	-	5%

9) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	-	12%
30	Nonionic surfactant	1	-	4%
	Soap as fatty acid	2	-	6%
	Sodium carbonate (as Na_2CO_3)	14	-	22%
	Zeolite (as NaAlSiO_4)	18	-	32%
	Sodium sulfate (as Na_2SO_4)	5	-	20%
35	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	3	-	8%

	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$)	4	-	9%
	Bleach activator (e.g. NOBS or TAED)	1	-	5%
	Carboxymethylcellulose	0	-	2%
5	Polymers (e.g. polycarboxylate or PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
10	Minor ingredients (e.g. optical brightener, perfume)	0	-	5%

10) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	-	23%
15	Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8	-	15%
	Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3	-	9%
20	Soap as fatty acid (e.g. lauric acid)	0	-	3%
	Aminoethanol	1	-	5%
	Sodium citrate	5	-	10%
	Hydrotrope (e.g. sodium toluenesulfonate)	2	-	6%
25	Borate (as B_4O_7)	0	-	2%
	Carboxymethylcellulose	0	-	1%
	Ethanol	1	-	3%
	Propylene glycol	2	-	5%
30	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	-	5%

11) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	20	- 32%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	6	- 12%
	Aminoethanol	2	- 6%
	Citric acid	8	- 14%
	Borate (as B ₄ O ₇)	1	- 3%
10	Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
15	Glycerol	3	- 8%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. hydro- tropes, dispersants, perfume, optical brighteners)	0	- 5%

12) A detergent composition formulated as a granulate having
a bulk density of at least 600 g/l comprising

25	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfa- te, alpha-olefinsulfonate, alpha- sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
30	Sodium carbonate (as Na ₂ CO ₃)	8	- 25%
	Soluble silicates (as Na ₂ O, 2SiO ₂)	5	- 15%
	Sodium sulfate (as Na ₂ SO ₄)	0	- 5%
	Zeolite (as NaAlSiO ₄)	15	- 28%
	Sodium perborate (as NaBO ₃ ·4H ₂ O)	0	- 20%
35	Bleach activator (TAED or NOBS)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

13) Detergent formulations as described in 1) - 12) where in all or part of the linear alkylbenzenesulfonate is replaced by (C₁₂-C₁₈) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C ₁₂ -C ₁₈) alkyl sulfate	9	- 15%
	Alcohol ethoxylate	3	- 6%
	Polyhydroxy alkyl fatty acid amide	1	- 5%
	Zeolite (as NaAlSiO ₄)	10	- 20%
10	Layered disilicate (e.g. SK56 from Hoechst)	10	- 20%
	Sodium carbonate (as Na ₂ CO ₃)	3	- 12%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	0	- 6%
	Sodium citrate	4	- 8%
15	Sodium percarbonate	13	- 22%
	TAED	3	- 8%
	Polymers (e.g. polycarboxylates and PVP=	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	- 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C ₁₂ -C ₁₈) alkyl sulfate	4	- 8%
	Alcohol ethoxylate	11	- 15%
	Soap	1	- 4%
	Zeolite MAP or zeolite A	35	- 45%
30	Sodium carbonate (as Na ₂ CO ₃)	2	- 8%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	0	- 4%
	Sodium percarbonate	13	- 22%
	TAED	1	- 8%

Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
5 Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an
10 additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9),
15 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous
20 detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxyated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The following examples further illustrate the
25 present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1**Bleaching of Direct Blue 1 with soybean peroxidase with and without acetosyringone**

A crude soy bean peroxidase (SBP), obtained from Mead
5 Corp., Dayton, Ohio, was purified by anion and cation chroma-
tography followed by gelfiltration to a single protein on
SDS-PAGE with an R_f -value (A_{404nm}/A_{280nm}) of 2.2:

125 ml of crude SBP were adjusted to pH 7, diluted to
2.3 mS and filtered through 0.8 μ filter. The sample was
10 applied to 300 ml DEAE column equilibrated with 20 mM
phosphate pH 7.0 and the peroxidase eluted with a 1 M NaCl
linear gradient in the same buffer. Fractions with peroxidase
activity were pooled.

Pooled fractions from anion exchange chromatography (190
15 ml) were concentrated and washed by ultrafiltration (GR61PP
membrane from Dow, Denmark). pH was adjusted to 5.3 ionic
strength to 2.3 mS in the sample before application to a 200
ml S-Sepharose column previously equilibrated with 50 mM
acetate pH 5.3. The effluent containing the peroxidase
20 activity was concentrated and washed by ultrafiltration to a
final volume of approx. 10 ml.

A 5 ml concentrated sample from cation exchange chroma-
tography was applied to a 90 cm Sephacryl S-200 column
equilibrated and eluted with 0.1 M acetate pH 6.1. Fractions
25 with peroxidase activity giving only one band on SDS-PAGE
were pooled.

The bleaching rate of Direct Blue 1 (DB1) by the
purified SBP was determined using an enhancer according to
the invention. The following conditions were used:

	Final concentration
200 μ l 50 mM Britton-Robinson buffer*	
pH 6, 8 and 10, respectively	10 mM
200 μ l DB1 ~ 3.0 Abs. Units (610 nm)	0.6 (A_{610nm})
5 200 μ l SBP with $A_{404nm} = 0.0005$ at pH 6	
and 8 or with $A_{404nm} = 0.005$ at pH 10	0.0001 or
	0.001 (A_{404nm})**
200 μ l 50 μ M enhancer	10 μ M
200 μ l 100 μ M H_2O_2	20 μ M

10 * (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

** corresponding to approximately to 0.04 mg/l and 0.4 mg/l.

Reagents were mixed in a thermostated cuvette at 30°C and the bleaching was started by addition of hydrogen
 15 peroxide. The bleaching was detected spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of DB1. Bleaching was followed for 4 minutes, and the reduction in absorbance ($100 \times (A_{610nm, start} - A_{610nm, 4min.}) / A_{610nm, start} \%$) was determined.

20 $A_{610nm, start}$ was determined by replacement of hydrogen peroxide with water.

Table 1

Bleaching of Direct Blue 1 with SBP in 4 Minutes

Enhancer	% DB1 bleaching in 4 min.		
	pH 6	pH 8	pH 10 10x[SBP]
25 No	0.7	<0.7	<0.7
acetosyringone	19.8	20.0	3.3

From the results presented in Table 1 above, it appears that by adding an enhancer of the invention a much faster bleaching of the dye is obtained compared to the
 30 experiment without enhancer.

EXAMPLE 2Bleaching of Direct Blue 1 with Coprinus cinereus peroxidase with and without enhancers

A Coprinus cinereus peroxidase (CiP) obtained as described in WO 9412621 was used.

Dilutions of CiP were made in a solution of 0.15 gram/l of Triton X-405.

The bleaching rate of Direct Blue 1 (DB1) by purified CiP was determined using the following conditions:

10		Final concentration
	200 μ l 50 mM Britton-Robinson buffer*	10 mM
	200 μ l DB1 - 3.0 Abs. Units (610 nm)	0.6 (A_{610nm})
	200 μ l 0.40 mg/l CiP (pH 8.5)	0.08 mg/l (pH 8.5) or
	0.80 mg/l CiP (pH 10.5)	0.16 mg/l (pH 10.5)
15	200 μ l 25 μ M enhancer	5 μ M
	200 μ l 100 μ M H_2O_2	20 μ M

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a thermostated cuvette at 20 30°C and the bleaching was started by addition of hydrogen peroxide. The bleaching was detected spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of DB1. Bleaching was followed for 1 minute, and the initial reduction in absorbance, $-\Delta mAbs/minute$, was determined.

Table 2Initial Bleaching of Direct Blue 1 with CiP

Enhancer	-ΔmAbs/minute	
	pH:	
	8.5	10.5
5 Acetosyringone	239	1
Syringaldehyde	151	4
Methylsyringate	245	8
No enhancer	2	0

From the results presented in Table 2 above it
10 appears that by adding an enhancer of the invention a much
faster bleaching of the dye compared to the experiment
without enhancer is obtained. Even at pH 10.5 a significant
bleaching with an enhancer of the invention is obtained,
whereas no bleaching at all can be seen without the addition
15 of an enhancer.

EXAMPLE 3Bleaching of Chicago Sky Blue 6B (CSB) with *Coprinus cinereus*
peroxidase and enhancers

Bleaching tests were performed in exactly the same way
20 as described in Example 2 except that instead of using DB1
Chicago Sky Blue (CSB) (obtainable from Aldrich) was used,
and the following enhancers were tested:
methylsyringate
ethylsyringate
25 propylsyringate
butylsyringate
hexylsyringate
octylsyringate
ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate.

30 The following results were obtained:

Table 3Initial Bleaching of CSB with CiP

	Enhancer	-ΔmAbs/minute	
		pH: 8.5	10.5
5	methylsyringate	211	42
	ethylsyringate	240	52
	propylsyringate	228	60
	butylsyringate	228	48
	hexylsyringate	276	36
10	octylsyringate	192	15
	ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate	48	48
	No enhancer	8	6

15 **EXAMPLE 4**

Bleaching of Direct Blue 1 (DB1) using various Coprinaceae laccases and methylsyringate at pH 5.5-8.5.

Bleaching of the dye Direct Blue 1 at various pH values was conducted using a laccase obtained from Coprinus comatus,
 20 Coprinus friesii, Coprinus plicatilis, Panaeolus papilionaceus or Psathyrella condolleana and methylsyringate.

The above mentioned strains were fermented in the following way:

The strains were inoculated on PDA agar plates (PDA: 39
 25 g/l potato dextrose agar) and grown at 26°C for 3 days. Shake flasks were then inoculated with 6-8 small squares (~0.5 cm x 0.5 cm) of agar containing mycelium and fermented for 3-10 days at 26°C and 200 rpm using the following medium:

	Deposit no.	Medium	Growth
<u>Coprinus comatus*</u>	CBS 631.95	A	10 days
<u>Coprinus friesii</u>	CBS 629.95	A	3 days
<u>Panaeolus</u>			
5 <u>papilionaceus</u>	CBS 630.95	A	10 days
<u>Psathyrella</u>			
<u>condolleana</u>	CBS 628.95	B	7 days
<u>Coprinus plicatilis</u>	CBS 627.95	A	8 days

* All the strains mentioned in this Example have been
10 deposited according to the Budapest Treaty on the Inter-
national Recognition of the Deposits of Microorganisms for
the Purpose of Patent Procedures, on 16 August 1995, at
Centraalbureau voor Schimmelcultures, Oosterstraat 1, Postbus
273, NL-3740 AG Baarn, Netherlands, under the above mentioned
15 Accession numbers.

Media:

A: soja meal 30 g/l
maltodextrin 15 g/l
bacto peptone 5 g/l
20 pluronic 0.2 g/l

B: potato meal 50 g/l
barley meal 25 g/l
BAN 800MG* 0.025 g/l
Na-caseinate 5 g/l
25 crushed soja 10 g/l
Na₂HPO₄, 12 H₂O 4.5 g/l
Pluronic 0.05 ml/l

* BAN 800MG obtainable from Novo Nordisk A/S.

After fermentation the culture broths were centrifugated
30 and the supernatants were used in the tests described below.

The bleaching rate of DB1 was determined using the
following conditions:

	Final concentration
400 μ l 50 mM Britton-Robinson buffer*, (pH 5.5, 7.0, and 8.5 respectively),	20 mM
200 μ l DB1 ~ 3.0 Abs. Units (610 nm)	0.6 (A_{610nm})
5 200 μ l 50 μ M methylsyringate	10 μ M
200 μ l laccase	at pH 5 and 7: 4 LACU/l at pH 8.5: 20 LACU/l

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

10 Reagents were mixed in a 1 ml thermostated cuvette at 30°C and the bleaching was started by addition of the laccase.

The bleaching was followed spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of
15 DB1, with readings every 5 sec. for a period of 5 minutes. The initial bleaching rate was determined from the first linear part of the absorbance curve.

The following results were obtained with methylsyringate:

	- Δ mAbs/minute
Laccase:	
pH:	5.5 7.0 8.5
C. comatus	33 23 2
25 C. friesii	40 55 61
Pan. papilionaceus	16 19 18
Ps. condolleana	45 54 43
C. pliccalitis	42 39 14

The following results were obtained with no
30 enhancer:

		-ΔmAbs/minute		
Laccase:				
	pH:	5.5	7.0	8.5
5	C. comatus	0	0	0
	C. friesii	0	0	0
	Ps. condolleana	0	0	0
	C. pliccalitis	0	0	0

EXAMPLE 5

10 Bleaching of Direct Blue 1 (DB1) using *Coprinus cinereus* laccase with/without enhancing agents at pH 5.5-8.5.

Bleaching of the dye Direct Blue 1 at various pH values was conducted using *Coprinus cinereus* laccase and one of the following enhancing agents:

15 None

acetosyringone
syringaldehyde
methoxysyringate.

The laccase was obtained in the following way:

20 *Coprinus cinereus* (IFO 30116 - freely available to the public from Institute of Fermentation, Osaka (IFO) under the indicated deposit number) was inoculated from a PDA agar slant (PDA: 39 g/l potato dextrose agar) into a 100 ml shake flask containing medium A (Medium A is described in Example 25 3). The culture was cultivated for 6 days at 26°C and 100 rpm. A 10-liter fermentor containing medium A was inoculated with the 100 ml culture broth. The fermentation ran for 6 days at 26°C and 100 rpm. The culture broth was filtrated and concentrated by ultrafiltration. Further purification was 30 carried out using hydrophobic interaction chromatography followed by anionic exchange chromatography. This process resultated in a preparation with a laccase activity of 3.6 LACU/ml. The estimated purity was >80% on a protein basis.

The bleaching rate of DB1 was determined using the following conditions:

	Final concentration
400 μ l 50 mM Britton-Robinson buffer*,	
5 (pH 5.5, 7.0 and 8.5 respectively),	20 mM
200 μ l DB1 ~ 3.0 Abs. Units (610 nm)	0.6 (A_{610nm})
200 μ l 50 μ M enhancing agent	10 μ M
200 μ l <u>C. cinereus</u> laccase	1 mg/l

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric
10 acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a 1 ml thermostated cuvette at 30°C and the bleaching was started by addition of the laccase.

The bleaching was followed spectrophotometrically at
15 610 nm, which is the wavelength of the absorption peak of DB1, with readings every 5 sec. for a period of 5 minutes. The initial bleaching rate was determined from the first linear part of the absorbance curve.

The following results were obtained:

20 Enhancing agent	- Δ Abs/minute		
pH:	5.5	7.0	8.5
none	13	5	3
aceto-			
25 syringone	28	94	50
syring-			
aldehyde	29	79	28
methyl-			
syringate	20	94	57

EXAMPLE 6Bleaching of Direct Blue 1 (DB1) using *Coprinus cinereus* laccase and acetosyringone

Bleaching of the dye Direct Blue 1 at various pH values was conducted using *Coprinus cinereus* laccase and the enhancing agent acetosyringone.

The laccase was obtained as described in Example 5.

The bleaching rate of DB1 was determined using the following conditions:

	Final concentration
400 μ l 50 mM Britton-Robinson buffer*, (pH 4, 5, 6, 7, and 8 respectively),	20 mM
200 μ l DB1 ~ 3.0 Abs. Units (610 nm)	0.6 (A_{610nm})
200 μ l 50 μ M acetosyringone	10 μ M
200 μ l <i>C. cinereus</i> laccase	3.2 mg/l

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a 1 cm thermostated cuvette at 30°C and the bleaching was started by addition of the laccase.

The bleaching was detected spectrophotometrically at 610 nm, which is the wavelength of the absorption peak of DB1. After 5 sec. bleaching was followed for 4 minutes.

The following results were obtained:

pH	Initial DB1 bleaching ($-\Delta mAbs / \text{min}$) (% of pH 7-value)
4	18 %
5	13 %
6	35 %
7	100 %
8	69 %

It can be seen from the results given above that the optimum bleaching is achieved at pH around 7, but the system also shows an effective bleaching at pH 8.

EXAMPLE 7

5 Bleaching of Direct Blue 1 with *Trametes villosa* laccase with and without enhancing agents

Laccase obtained from *Trametes villosa*: 800 ml culture broth of *Trametes villosa*, CBS 678.70, was filtered with filter aid to give a clear filtrate, which was concentrated and washed
10 by ultrafiltration on a membrane with a cut-off of 6-8 kDa. One ml samples of concentrated preparation was applied onto a Q-Sepharose HP column (Pharmacia, Sweden) equilibrated with 0.1 M fosfate pH 7, and the laccase was eluted with a flat NaCl gradient around 0.25 M. Fractions with laccase activity
15 from 10 runs were pooled and concentrated by ultrafiltration to an activity of 500 LACU/ml.

The following conditions were used:

		Final concentration
20	400 μ l 50 mM Britton-Robinson buffer*,	
	pH 5.5 and pH 7.0 respectively,	20 mM
	200 μ l DB1 ~ 3.0 Abs. Units (610 nm)	0.6 (A_{610nm})
	200 μ l 50 μ M enhancer	10 μ M
	200 μ l Enzyme dilution	

* (50 mM acetic acid, 50 mM phosphoric acid, 50 mM
25 boric acid, pH adjusted to the value of interest with NaOH).

Reagents were mixed in a 1 cm thermostated cuvette at 30°C and the bleaching was started by addition of enzyme.

The bleaching was detected spectrophotometrically at 610 nm, which is the absorption peak of DB1. After 5 sec.
30 bleaching was followed for 4 minutes.

From the results presented below, it appears that adding enhancers of the invention a much faster bleaching of

the dye can be obtained compared to the experiment without enhancer. Enzyme dosages given are in the final incubation mixture.

Bleaching of Direct Blue 1 with Trametes villosa laccase, obtained as described above, at pH 5.5 (1.6 mg/l) and pH 7.0 (16 mg/l):

Enhancer	DB1 bleaching in 4 minutes ($-\Delta mAbs/4 \text{ min}$)	
	pH 5.5	pH 7.0
No enhancer	0	0
Acetosyringone	447	242
Syringaldehyde	438	112

EXAMPLE 8

Bleaching of gradually added Acid Blue 45 with Coprinus cinereus laccase with and without enhancing agent

Ideally, dye transfer inhibition systems for laundry applications should be tested in a real wash where dyed fabrics give off dyes to the wash solution as a result of the combined action of the detergent, temperature and mechanical agitation taking place.

To simulate such a process, however, a magnetically stirred beaker was used as the reaction vessel and dye was added gradually from a stock solution (using a Metrohm 725 dosimat). The solution was monitored spectrophotometrically using a Zeiss multichannel spectrometer (MCS) equipped with a fibre-optics immersion probe.

Stock solutions of acetosyringone was prepared in a suitable water/ethanol mixture. Stock solutions of the anthraquinone dye Acid Blue 45 were made with water.

The laccase was recovered from a 10-lit r fermentation of Coprinus cinereus (IFO 30116) as described in Example 4.

The following conditions were used in the
5 experiment:

Temperature: 35°C

Medium and pH: 50 mM/50 mM phosphate/borate buffer at pH 10

Acetosyringone (when applicable): 10 µM

Laccase: 10 mg/l

10 Dye addition program: linear addition at a rate of ca 0.34 abs/40 min, referring to the absorbance of Acid Blue 45 at its maximum absorbance wavelength (590 nm for Acid Blue 45).

Fig. 1 shows the results of the bleaching tests.

The following symbols are used: (I): Only dye addition; (II):
15 Dye addition in the presence of Laccase; (III): Dye addition in the presence of Laccase + acetosyringone.

It can be seen from Fig. 1 that the bleaching effect is enhanced by acetosyringone.

EXAMPLE 9

20 Dye Transfer Inhibition Using Coprinus cinereus Laccase

A small-scale experiment was carried out in which clean cotton test pieces were washed together with dyed fabrics bleeding dye into the wash solution, the experiment conducted in the absence and in the presence of laccase and
25 enhancing agent.

After wash, the Hunter colour difference between the above mentioned cotton pieces and clean cotton pieces (washed in the absence of bleeding fabrics) was measured and taken as a measure of the degree of dye transfer resulting
30 from the wash.

Materials used:

Bleeding fabrics dyed with Acid Red 151 (AR 151) or Direct Blu 1 (DB1).

Clean white cotton (bleached, no optical brightener added).

Liquid detergent and powder detergent as typically met in the North American market place; both detergents contained no bleaching system.

Coprinus cinereus laccase, obtained as described in Example 4.

Washing procedure:

The washing processes were carried out in beakers with magnetical stirring at 35°C for 15 min., after which the test fabrics were rinsed thoroughly in tap water and air-dried overnight in the dark before the Hunter readings were taken by using a Datacolor Elrephometer 2000 reflectance spectrometer.

Laccase system: Laccase at a level of 10 mg/l with the enhancing agent acetosyringone at a level of 10 µM.

The following results were obtained:

Wash in liquid detergent solution (2 g/l, water hardness 6°dH) at pH 8.5:

	Hunter colour difference (delta E) with respect to white, washed cotton	
	Cotton washed with AR 151 bleeders	Cotton washed with DB 1 bleeders
Wash with no laccase system	12	26
Wash with laccase system	1	7

Wash in powder detergent solution (1 g/l, water hardness 6°dH) at pH 10.0:

Hunter colour difference (delta E)
with respect to white, washed cotton

5	Cotton washed with AR 151 bleeders	Cotton washed with DB 1 bleeders
---	--	--

	Wash with no laccase	
10	system	21 29
	Wash with laccase	
	system	4 8

Typical significant differences in the delta E readings are 2-3 units, so the data reflect significant reduction of dye transfer with the laccase treatments relative to the treatment with no laccase system.

EXAMPLE 10

Dye Transfer Inhibition Using Myceliophthora thermophila Laccase

20 A small-scale experiment was carried out in which clean cotton test pieces were washed together with dyed fabrics bleeding dye into the wash solution, the experiment conducted in the absence and in the presence of laccase and enhancing agent.

25 After wash, the Hunter colour difference between the above mentioned cotton pieces and clean cotton pieces (washed in the absence of bleeding fabrics) was measured and taken as a measure of the degree of dye transfer resulting from the wash.

Materials used:

Bleeding fabrics dyed with Acid Red 151 (AR 151) or Direct Blue 1 (DB1).

Clean white cotton (bleached, no optical brightener added).

Liquid detergent (No. 1) as typically met in the European market place; liquid detergent (No. 2) as typically met in the North American market place.

Myceliophthora thermophila laccase, produced as described in PCT/US95/06815).

Washing procedure:

The washing processes were carried out in beakers with magnetical stirring at 35°C for 15 min., after which the test fabrics were rinsed thoroughly in tap water and air-dried overnight in the dark before the Hunter readings were taken by using a Datacolor Elrephometer 2000 reflectance spectrometer.

Laccase systems: M. thermophila laccase at a level of 0.87 mg/l with the enhancing agent acetosyringone (AS) or the enhancing agent methylsyringate (MS) at a level of 10 µM.

The following results were obtained:

Wash in solution of liquid detergent No. 1 (7 g/l, water hardness 12°dH) at an initial pH of 7.0:

Hunter colour difference (delta E)
with respect to white, washed cotton

Cotton washed
with AR 151
bleeders

Cotton washed
with DB 1
bleeders

Wash with no laccase
system

40

Wash with AS-based laccase system	5	13
Wash with MS-based laccase system	4	12

5 Wash in solution of liquid detergent No. 2 (2 g/l, water hardness 6°dH) at pH 8.5:

Hunter colour difference (delta E)
with respect to white, washed cotton

10	Cotton washed with AR 151 bleeders	Cotton washed with DB 1 bleeders
----	--	--

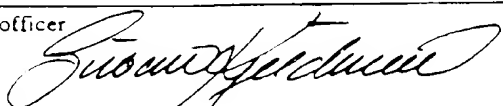
Wash with no laccase system	14	29
15 Wash with AS-based laccase system	5	10
Wash with MS-based laccase system	3	8

Typical significant differences in the delta E readings are
20 2-3 units, so the data reflect significant reduction of dye transfer with the laccase treatments relative to the treatment with no laccase system.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>29</u> , lines <u>2</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
Address of depositary institution (including postal code and country) Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Nether- land	
Date of deposit 16 August 1995	Accession Number CBS 631.95
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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(PCT Rule 13bis)


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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution <p style="text-align: center;">CENTRAALBUREAU VOOR SCHIMMELCULTURES</p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;">Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Nether- land</p>	
Date of deposit <p style="text-align: center;">16 August 1995</p>	Accession Number <p style="text-align: center;">CBS 629.95</p>
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution <p style="text-align: center;">CENTRAALBUREAU VOOR SCHIMMELCULTURES</p>	
Address of depositary institution (including postal code and country) <p style="text-align: center;">Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Nether- land</p>	
Date of deposit <p style="text-align: center;">16 August 1995</p>	Accession Number <p style="text-align: center;">CBS 628.95</p>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
<p>The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")</p>	

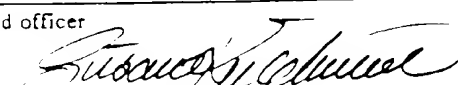
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

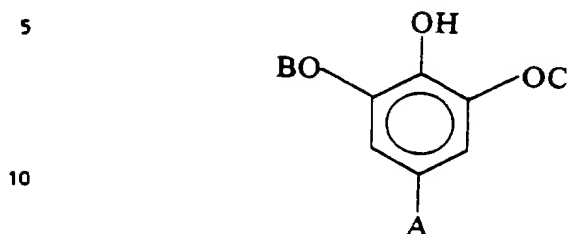
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>29</u> , lines <u>8</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution CENTRAALBUREAU VOOR SCHIMMELCULTURES	
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Date of deposit 16 August 1995	Accession Number CBS 627.95
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
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D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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CLAIMS

1. A method of oxidizing a compound with a phenol oxidizing enzyme, characterized by the presence of an enhancing agent of the following formula:



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, -SO₂-E, -N-XY, and
 15 -N⁺-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C₁-C₁₆ alkyl, preferably a C₁-C₈ alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino
 20 group; and B and C may be the same or different and selected from C_mH_{2m+1}; 1 ≤ m ≤ 5.

2. A method according to claim 1, in which the enhancing agent is selected from the group consisting of acetosyringone, syringaldehyde, methylsyringate and syringic
 25 acid.

3. A method according to claims 1-2, in which the phenol oxidizing enzyme is a peroxidase and a hydrogen peroxide source.

4. A method according to claim 3, wherein the
 30 peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from Coprinus, e.g. C. cinereus or C. macrorrhizus, or from Bacillus, e.g. B. pumilus, or Myxococcus, e.g. M. virescens.

5. A method according to claim 3 or 4, wherein the hydrogen peroxidase source is hydrogen peroxide or a hydrogen peroxide precursor, e.g. perborate or percarbonate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and its substrate, or a peroxycarboxylic acid or a salt thereof.

6. A method according to claim 1, in which the phenol oxidizing enzyme is a laccase or a laccase related enzyme together with oxygen.

10 7. A method according to claim 6, wherein the laccase is derived from Trametes, e.g. Trametes villosa, or Coprinus, e.g. Coprinus cinereus, or bilirubin oxidase derived from Myrothecium, e.g. M. verrucaria.

8. A method according to any of claims 1-7, in
15 which said method is a method for bleaching dyes in solution.

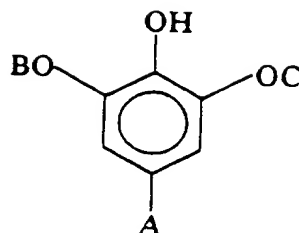
9. A method according to any of claims 1-8, in which said method is a method for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor.

20 10. A method according to claims 8-9, in which the enhancing agent is added at the beginning of, or during the process.

11. A method according to any of claims 8-10, in which the concentration of the enhancing agent is in the
25 range of from 0.01-1000 μM , more preferred 0.1-250 μM , most preferred 1-100 μM .

12. A detergent additive comprising a phenol oxidizing enzyme and an enhancing agent of the formula

5



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected
 10 from the group consisting of -CO-E, -SO₂-E, -N-XY, and -N⁺-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C₁-C₁₆ alkyl, preferably a C₁-C₈ alkyl, which alkyl may be saturated or unsaturated, branched or unbranched
 15 and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m+1}; 1 ≤ m ≤ 5.

13. A detergent additive according to claim 12, in which the enhancing agent is selected from the group
 20 consisting of acetosyringone, syringaldehyde, methylsyringate and syringic acid.

14. A detergent additive according to claims 12-13, in which the phenol oxidizing enzyme is a peroxidase and a hydrogen peroxide source.

25 15. A detergent additive according to claim 14, wherein the peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from Coprinus, e.g. C. cinereus or C. macrorrhizus, or from Bacillus, e.g. B. pumilus, or Myxococcus, e.g. M. virescens.

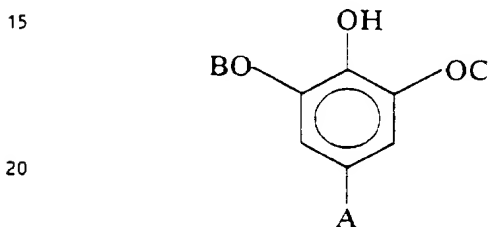
30 16. A detergent additive according to claims 14-15, wherein the hydrogen peroxide source is hydrogen peroxide or a hydrogen peroxide precursor, e.g. perborate or percarbonate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and a suitable substrate, or a per-
 35 oxycarboxylic acid or a salt thereof.

17. A detergent additive according to claim 12, in which the phenol oxidizing enzyme is a laccase or a laccase related enzyme together with oxygen.

18. A detergent additive according to claim 17, wherein the laccase is derived from Trametes, e.g. Trametes villosa, or Coprinus, e.g. Coprinus cinereus, or bilirubin oxidase derived from Myrothecium, e.g. M. verrucaria.

19. A detergent additive according to any of claims 12-18, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

20. A detergent composition comprising a phenol oxidizing enzyme, a surfactant and an enhancing agent of the formula



in which formula A is a group such as -D, -CH=CH-D, -CH=CH-CH=CH-D, -CH=N-D, -N=N-D, or -N=CH-D, in which D is selected from the group consisting of -CO-E, -SO₂-E, -N-XY, and -N⁺-XYZ, in which E may be -H, -OH, -R, or -OR, and X and Y and Z may be identical or different and selected from -H and -R; R being a C₁-C₁₆ alkyl, preferably a C₁-C₈ alkyl, which alkyl may be saturated or unsaturated, branched or unbranched and optionally substituted with a carboxy, sulfo or amino group; and B and C may be the same or different and selected from C_mH_{2m+1}; 1 ≤ m ≤ 5.

21. A detergent composition according to claim 20, in which the enhancing agent is selected from the group con-

sisting of acetosyringone, syringaldehyde, methylsyringate and syringic acid.

22. A detergent composition according to claim 21, in which the phenol oxidizing enzyme is a peroxidase and a hydrogen peroxide source.

23. A detergent composition according to claim 22, wherein the peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from Coprinus, e.g. C. cinereus or C. macrorrhizus, or from Bacillus, e.g. B. pumilus, or Myxococcus, e.g. M. virescens.

24. A detergent composition according to claims 22-23, wherein the hydrogen peroxide source is hydrogen peroxide or a hydrogen peroxide precursor, e.g. perborate or percarbonate, or a hydrogen peroxide generating enzyme system, e.g. an oxidase and its substrate, or a peroxy-carboxylic acid or a salt thereof.

25. A detergent composition according to claim 20, in which the phenol oxidizing enzyme is a laccase or a laccase related enzyme together with oxygen.

26. A detergent composition according to claim 25, wherein the laccase is derived from Trametes, e.g. Trametes villosa, or Coprinus, e.g. Coprinus cinereus, or bilirubin oxidase derived from Myrothecium, e.g. M. verrucaria.

27. A detergent composition according to any of claims 20-26, which further comprises one or more other enzymes, in particular a protease, a lipase, an amylase, a cellulase, and/or a cutinase.

28. A method according to any of claims 1-7, in which said method is a method for bleaching of lignin-containing material, in particular bleaching of pulp for paper production.

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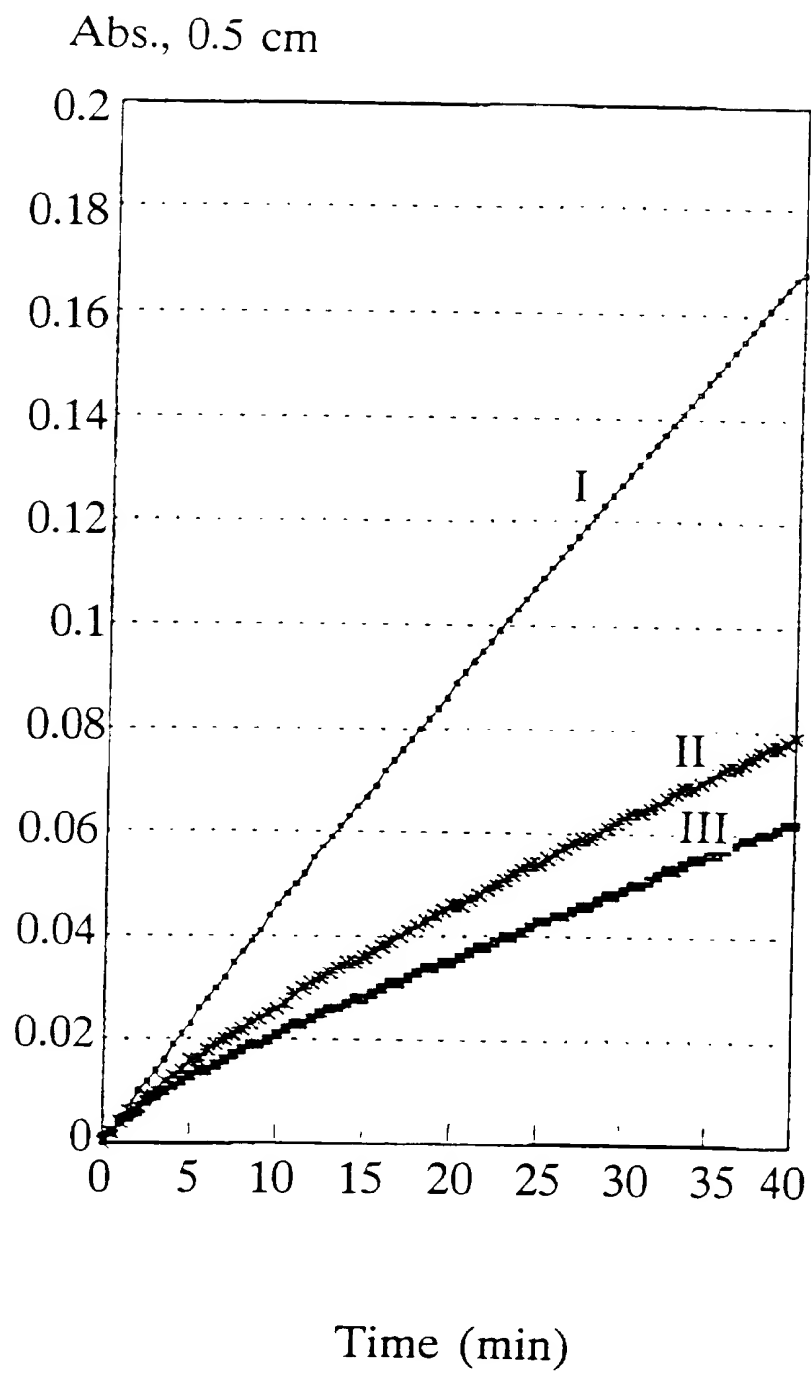


FIG. 1

INTERNATIONAL SEARCH REPORT

1

International application No.

PCT/DK 95/00384

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/02, C11D 3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, BIOSIS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Enzyme Microb. Technol., Volume 8, March 1986, Kay L. Shuttleworth et al, "Soluble and immobilized laccase as catalysts for the transformation of substituted phenols", page 171, see abstract --	1-28
X	Wood research., Volume 76, 1989, Shingo Kawai et al, "Oxidation of Methoxylated Benzyl Alcohols by Laccase of Coriolus versicolor in the Presence of Syringaldehyde", page 10 - page 11, see abstract --	1-28

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

24 January 1996

Date of mailing of the international search report

07-02-1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00384

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 123, No 22, 27 November 1995 (27.11.95), (Columbus, Ohio, USA), Roper, J. Chadwick et al, "Enhanced enzymic removal of chlorophenols in the presence of co-substrates", page 677, THE ABSTRACT No 295447h, Water Res. 1995, 29 (12), 2720-2724 --	1-28
A	WO 9412621 A1 (NOVO NORDISK), 9 June 1994 (09.06.94) --	1-28
A	Chemical Abstracts, Volume 112, No 21, 21 May 1990 (21.05.90), (Columbus, Ohio, USA), Pekarovicova, Alexandra et al, "An activation effect of some phenolics on the enzymic hydrolysis of polysaccharides", page 318, THE ABSTRACT No 194308, Cellul. Chem. Technol. 1989, 23 (3), 225-233 -- -----	1-28

Information on patent family members

PCT/DK 95/00384

Patent document
cited in search report

Patent family member(s)

Publication
date

NONE